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DEVELOPMENT AND DISEASE

Pin1 regulates the timing of mammalian primordial germ cell proliferation

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SUMMARY

Primordial germ cells (PGCs) give rise to male and female germ cells to transmit the genome from generation to generation. Defects in PGC development often result in infertility. In the mouse embryo, PGCs undergo proliferation and expansion during and after their migration to the gonads from 8.5 to 13.5 days post coitum (dpc). We show that a peptidyl-prolyl isomerase, Pin1, is involved in the regulation of mammalian PGC proliferation. We discovered that both the male and female *Pin1*$^{-/-}$ mice had profound fertility defects. Investigation of the reproductive organs revealed significantly fewer germ cells in the adult *Pin1*$^{-/-}$ testes and ovaries than in wild type or heterozygotes, which resulted from *Pin1*$^{-/-}$ males and females being born with severely reduced number of gonocytes and oocytes. Further studies in 8.5 to 13.5 dpc *Pin1*$^{-/-}$ embryos showed that PGCs were allocated properly at the base of the allantois, but their cell expansion was progressively impaired, resulting in a markedly reduced number of PGCs at 13.5 dpc. Analyses using markers of cell cycle parameters and apoptosis revealed that *Pin1*$^{-/-}$ PGCs did not undergo cell cycle arrest or apoptosis. Instead, *Pin1*$^{-/-}$ PGCs had a lower BrdU labeling index compared with wild-type PGCs. We conclude that PGCs have a prolonged cell cycle in the absence of *Pin1*, which translates into fewer cell divisions and strikingly fewer *Pin1*$^{-/-}$ PGCs by the end of the proliferative phase. These results indicate that Pin1 regulates the timing of PGC proliferation during mouse embryonic development.

Key words: Primordial germ cells, Pin1, Proliferation, Cell cycle, Knockout mice

Supplementary figures available online

INTRODUCTION

In the mouse, primordial germ cells (PGCs) are established from precursors in the epiblast at the base of the future allantois at 7.5 dpc (Lawson and Hage, 1994; McLaren, 2000; Saitou et al., 2002). Starting at 8.5 dpc, PGCs begin to migrate through the hindgut and dorsal mesentery, and arrive in the embryonic gonads by 10.5 dpc (Gomperts et al., 1994; Wylie, 1999). During and after PGC migration, they proliferate and increase rapidly in number until 13.5 dpc (McLaren, 2000; Tam and Snow, 1981). After this time, PGCs in the male (XY) gonad undergo cell cycle arrest until a few days after birth, whereas in the female (XX) gonad, PGCs enter meiosis and arrest in prophase of meiosis I (McLaren, 2000). A number of extracellular growth factors and receptors, including Stem cell factor (Scf), Fgf and Kit, have been shown to play a role in PGC survival and proliferation (De Miguel et al., 2002; Donovan, 1998; Matsui et al., 1992; Resnick et al., 1992). However, the intracellular events regulating PGC proliferation are currently not well understood. Because very few PGCs can be isolated and because of the technical limitations in the manipulation of gene expression in these cells in vitro (De Miguel et al., 2002; Watanabe et al., 1997), intracellular factors involved in the regulation of PGC development often are discovered in spontaneous and targeted mouse genetic models (Agoulnik et al., 2002; Pellas et al., 1991; Takeuchi et al., 2003). We identify a peptidyl-prolyl isomerase, Pin1, as a regulator of the timing of PGC proliferation using a targeted knockout mouse model.

Pin1 catalyses the cis-trans isomerization of phosphorylated serine/threonine-proline bonds in phosphoproteins, thereby altering conformation leading to a change in protein stability or function (Lu et al., 2002; Stukenberg and Kirschner, 2001; Yaffe et al., 1997). Many studies in cultured cells have implicated Pin1 as a regulator of cell cycle progression, as well as the DNA replication and DNA damage checkpoints (Lu et al., 1996; Lu et al., 2002; Winkler et al., 2000; Zacchi et al., 2002; Zheng et al., 2002). Pin1 has been shown to interact with, and suggested to regulate, crucial cell signaling proteins such as Jun, cyclin D1, Cdc25, β-catenin and p53 (Crenshaw et al., 1998; Liou et al., 2002; Ryo et al., 2001; Wulf et al., 2001; Zacchi et al., 2002; Zheng et al., 2002). In addition, Pin1 plays a role in dorsoventral patterning of the developing egg chamber in *Drosophila* by regulating the stability of a transcription factor, Cf2, via the MAPK pathway (Hsu et al., 2001). Depletion of Pin1 in HeLa cells and budding yeast has been

reported to cause mitotic arrest and nuclear fragmentation in those cells (Lu et al., 1996). In spite of the numerous critical cellular roles attributed to Pin1 (Lu et al., 2002), adult mice homozygous for the targeted deletion of the *Pin1* gene were reported to exhibit only mild defects in the mammary gland, and in testis and retina of very old animals (Liou et al., 2002). However, these studies were carried out on a mixed genetic background. To explore further the in vivo function of *Pin1*, we used marker-assisted speed congenic breeding to backcross the mutation into an inbred C57BL/6J background, in which the studies described below were conducted (see Fig. S1 at http://dev.biologists.org/supplemental/). In this report, we found that male and female *Pin1*$^{-/-}$ mice were born with fewer germ cells, resulting in severe fertility defects in both genders. We examined the development of PGCs in *Pin1*$^{-/-}$ embryos, and identified Pin1 as a regulator of the timing of PGC proliferation.

MATERIALS AND METHODS

Generation of *Pin1*$^{-/-}$ mice

Homozygous *Pin1*-mutant mice, originally generated by Fujimori et. al. (Fujimori et al., 1999), were obtained from Hoffman LaRoche (Nutley, NJ). The *Pin1* gene deletion was transferred into an isogenic C57BL/6J background using marker-assisted speed congenic protocols by the Jackson Laboratory. Genotyping was performed by PCR, using primers 5′-ATCATCCTGCGCACAGAATG-3′ and 5′-TCAATTCCTCCAGAAGGAGC-3′ for the wild-type *Pin1* allele, 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′ for the disrupted allele. Embryos were sex-typed by PCR for the *Smcy* and *Smcx* genes using primers 5′-CCGCTGCCAAATTCTTTGG-3′ and 5′-TGAAGCTTTTGGCTTTGAG-3′.

Fertility studies

Continuous mating studies were carried out to assess fertility as described by others (Jeffs et al., 2001). Briefly, males around 12 weeks of age were housed individually with females around 7 weeks of age. Mounting behavior and copulatory plugs were observed to confirm normal mating behavior. Six pairs of each mating combination (wild-type males and females, *Pin1*$^{-/-}$ males and females, *Pin1*$^{-/-}$ males and wild-type females, *Pin1*$^{-/-}$ females and wild-type males) were followed for six months. The number of litters produced by each pair and the number of pups per litter were recorded and summed for each mating group.

Histology and immunohistochemistry

Postnatal testes and ovaries were fixed in Bouin's fixative and embedded in paraffin wax. Testes and ovaries were sectioned at 7 μm intervals. Testis sections were stained with periodic acid-Schiff reagent and Hematoxylin (PAS-H) (Polyscientific). Ovary sections were stained with Hematoxylin and Eosin. Testis sections were also processed for immunohistochemistry as previously described (Enders and May, 1994). Briefly, sections were deparaffinized, rehydrated, followed by antigen retrieval in 10 mM sodium citrate buffer. Sections were blocked in normal goat serum in PBS for 1 hour, and incubated with primary antibody at 4°C overnight. After washing in PBS, secondary antibody was applied for 1 hour and followed by washing in PBS. Staining was visualized using VectaStain (Vector Laboratories). Gonocytes were detected using the anti-GCNA1 antibody (a generous gift from G. C. Enders). Pin1 protein was detected using the rabbit polyclonal anti-Pin1 antibody 1:100 generated previously in our laboratory (Winkler et al., 2000). Specificity for the Pin1 immunostaining were confirmed in negative controls using the *Pin1*$^{-/-}$ tissues or pre-absorbed anti-Pin1 antibodies with GST-Pin1 proteins described previously in our laboratory (Winkler et al., 2000), and both provided similar negative staining results. Pin1 immunostaining in gonads was carried out in wholemounts as described below. For Pin1 immunostaining in 7.5, 8.5 and 9.5 dpc embryos, 10 μm embryo cryosections (see below) were blocked in blocking buffer (10% heat-inactivated goat serum, 3% BSA, 0.1% TritonX-100 in PBS) at room temperature for 2 hours, then incubated in anti-Pin1 antibodies (1:100 diluted in blocking buffer) overnight at 4°C. Sections were washed in wash buffer (1% heat-inactivated goat serum, 3% BSA, 0.1% TritonX-100 in PBS) three times for 10 minutes, and incubated in FITC-conjugated donkey anti-rabbit secondary antibodies 1:500 (Jackson ImmunoResearch) for 1 hour at room temperature. Sections were washed in wash buffer three times for 10 minutes with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) 1 μg/ml (Sigma) added in the last wash to identify the nuclei. The adjacent serial sections were processed for alkaline phosphatase assays to detect PGCs in those stages as described below.

PGC detection in embryos and gonads

Embryos were obtained from pregnant females 7.5, 8.5, 9.5, 11.5, 12.5 and 13.5 dpc, with the first day of vaginal plug identification defined as 0.5 dpc. Embryos and gonads were dissected in PBS and fixed in 4% paraformaldehyde overnight at 4°C, followed by washing in PBS. Embryos and gonads were ready to be processed as wholemounts after washing in PBS. For embryo sections, embryos were further dehydrated in 10% and 15% sucrose for 15 minutes each, and in 20% sucrose for 1 hour, then in 1:1 20% sucrose:OCT compound (Tissue-Tek) overnight at 4°C. Embryos were then embedded in 1:3 20% sucrose:OCT compound and cryosectioned at 10 μm. PGCs were detected in 7.5, 8.5 and 9.5 dpc embryos and in embryo sections using alkaline phosphatase assays as described by others (Lawson et al., 1999). Briefly, whole embryos or embryo sections were placed in 70% ethanol at 4°C for 1 hour, then washed once in PBS and stained with Fast Red TR and α-napthyl phosphate (Sigma) to detect alkaline phosphatase-positive cells. PGCs in 11.5, 12.5 and 13.5 wholemount gonads were detected using anti-PECAM 1:500 (Pharmingen) as previously described (Schmahl et al., 2000; Yao et al., 2002). Briefly, wholemount gonads were blocked for 3-4 hours in blocking buffer (1% heat-inactivated goat serum, 5% BSA, 0.1% TritonX-100 in PBS) at room temperature, then incubated in primary antibodies diluted in blocking buffer overnight at 4°C. Gonads were washed in wash buffer (0.1% TritonX-100 in PBS) three times for 1 hour at room temperature, and incubated in secondary antibodies in blocking buffer overnight at 4°C, followed by washing three times for 1 hour in wash buffer at room temperature and mounted on slides using imaging spacers (Sigma).

Apoptosis assay

Apoptotic cells were detected using LysoTracker Red (Molecular Probes) in wholemount gonads as previously described (Yao et al., 2002; Zucker et al., 1999). Briefly, 12.5 dpc gonads were dissected in sterile PBS, cultured in 500 μl DMEM medium with 2 μl/ml LysoTracker Red for 30 minutes in a 37°C, 5% CO_2 incubator. Gonads were washed in PBS, fixed in 4% paraformaldehyde overnight at 4°C and processed for whole-mount immunohistochemistry.

BrdU pulse labeling and antibodies

BrdU pulse labeling and detection were carried out using procedures previously described (Schmahl et al., 2000). Briefly, heterozygous pregnant females at 12.5 dpc received an i.p. injection of 50 mg/kg of BrdU (Sigma) and pulsed for 30 minutes. Gonads were dissected from embryos and processed for whole-mount immunohistochemistry as in Schmahl et al. (Schmahl et al., 2000). Anti-BrdU (Roche) 1:100, anti-Ki67 (Pharmingen) 1:100, anti-phosphohistone H3 (Upstate Biotechnology) 1:200 and anti-laminin (a generous gift from H.

Erickson) 1:200 were used accordingly. All conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:500 dilutions.

PGC quantitation

Embryos and gonads were mounted on slides using imaging spacers (Sigma) and cover slips after each experimental procedure. All PGCs in 8.5 and 9.5 dpc whole embryos were counted under a light microscope. PGCs in gonads were counted using methods in Schmahl et al. (Schmahl et al., 2000). Briefly, PGCs in the interior middle third segment of each gonad were counted using the 40× objective of a Zeiss LSM 410 confocal microscope, with images spaced at 15 μm intervals to avoid counting the same cells. The BrdU index was the ratio of BrdU-positive PGCs and all counted PGCs in each gonad.

Statistics

PGC numbers from each genotype, age and sex group were log-transformed because of heterogeneous variance across ages, and analysed using three-factor ANOVA (genotype, age, sex) in StatView. *P*-values were used to determine statistical significance. The BrdU index was analysed using unpaired Student's *t*-test. N for each category ranged from 6 to 19. Bar graphs were plotted in MS Excel.

RESULTS

Germ cell number deficiency in postnatal Pin1$^{-/-}$ mice

Both male and female *Pin1*-deficient mice were infertile when mated together. Over six months, six pairs of wild-type mice produced 35 litters and 279 pups, while no offspring were obtained from six pairs of *Pin1*$^{-/-}$ mice mated for the same time. No fertility defects were observed in heterozygous *Pin1*$^{+/-}$ males or females. To determine which gender was responsible for the infertility, we mated *Pin1*$^{-/-}$ males with wild-type females, and *Pin1*$^{-/-}$ females with wild-type males. We found that both *Pin1*$^{-/-}$ males and females were subfertile when mated with wild-type mice, producing only 12 litters and 63 pups by six pairs of *Pin1*$^{-/-}$ males, and 22 litters and 100 pups by six pairs of *Pin1*$^{-/-}$ females. The fewer number of litters and fewer pups per litter compared with wild-type matings for both genders indicated that both *Pin1*$^{-/-}$ males and females had fertility defects. To study the basis for the abnormal fertility, we examined the testes and ovaries of adult male and female *Pin1*$^{-/-}$ mice. In the wild-type testis, all seminiferous tubules contained germ cells organized in the multi-layered epithelium. Spermatogonia, spermatocytes and spermatids produced during the normal process of spermatogenesis were clearly identified (Fig. 1A). By contrast, the *Pin1*$^{-/-}$ testis had many seminiferous tubules that were markedly depleted of germ cells but did contain somatic Sertoli cells (Fig. 1B). Examination of adult ovaries showed multiple ovarian follicles in different stages of development in the wild-type ovary (Fig. 1C). However, very few follicles were seen in ovaries of *Pin1*$^{-/-}$ females (Fig. 1D). Because the male and female reproductive abnormalities were similar in that they both had very few germ cells, and because germ cells in the adult arise from postnatal gonocytes and oocytes, we speculated that there was a common germ cell defect in the early postnatal *Pin1*$^{-/-}$ males and females. Investigation of early postnatal testes and ovaries revealed that both males and females had a severely reduced number of germ cells at birth (Fig. 1E-H). In the newborn wild-type testes, gonocytes, identified by the germ cell nuclear antigen GCNA1 (Enders and May, 1994), populated the testis cords (Fig. 1E). However, *Pin1*$^{-/-}$ testis cords were either completely devoid of germ cells or contained fewer gonocytes (Fig. 1F). Formation of the testis cords appeared normal in the *Pin1*$^{-/-}$ testis. In newborn wild-type ovaries, multiple oocytes were present and had begun to induce formation of primordial ovarian follicles (Fig. 1G). By contrast, *Pin1*$^{-/-}$ ovaries had fewer oocytes and, as a consequence, few ovarian follicles were formed (Fig. 1H). These findings suggest that the development of primordial germ cells (PGCs), which give rise to the postnatal gonocytes and oocytes, is compromised in *Pin1*-deficient male and female embryos.

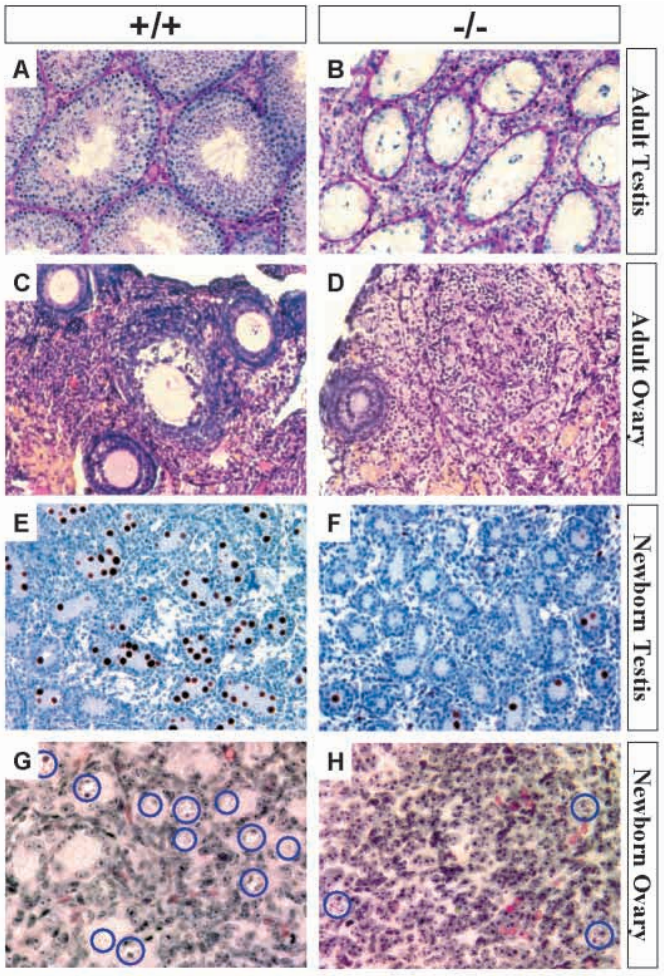

Fig. 1. Postnatal *Pin1*$^{-/-}$ males and females have fewer germ cells. (A,C,E,G) Wild-type; (B,D,F,H) *Pin1*$^{-/-}$. (A,B) Adult testis section shows that *Pin1*$^{-/-}$ testis has mostly empty seminiferous tubules containing only Sertoli cells and no germ cells. (C,D) Adult ovary section reveals that few ovarian follicles are seen in *Pin1*$^{-/-}$ females. (E,F) Newborn testis section shows that *Pin1*$^{-/-}$ testis has many fewer gonocytes, identified by the GCNA1 antigen (brown), within properly formed testis cords, while many gonocytes populate the wild-type testis. (G,H) Numerous oocytes, highlighted by the blue circles, are in a newborn wild-type ovary. The newborn *Pin1*$^{-/-}$ ovary has few oocytes. (A,B) Periodic acid-Schiff histochemistry. (C,D,G,H) Hematoxylin and Eosin. (E,F) Immunohistochemistry with antibodies to GCNA1.

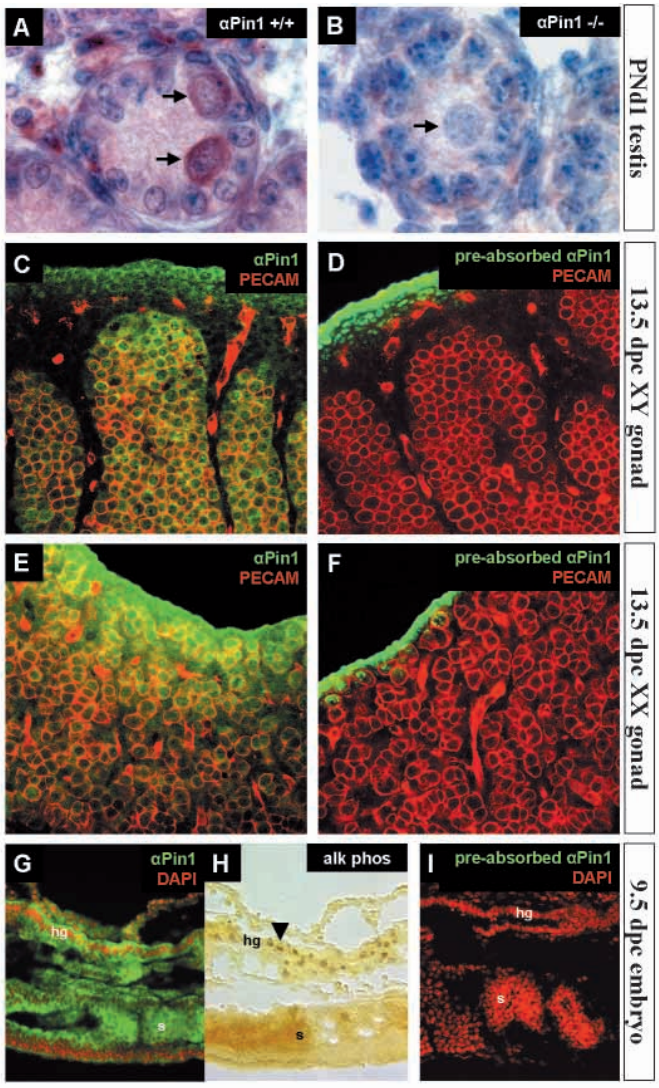

Fig. 2. Primordial germ cells express the Pin1 protein. (A) Immunohistochemistry using anti-Pin1 antibodies reveals high Pin1 protein expression in postnatal day 1 (P1) gonocytes (arrows). (B) No Pin1 protein is detected in *Pin1*$^{-/-}$ gonocytes (arrow). (C,E) Immunofluorescence shows intense Pin1 protein expression (green) in 13.5 dpc male XY (C) and female XX (E) PGCs, identified as large round cells by their surface antigen PECAM (red, round cells). (D,F) Negative control using pre-absorbed anti-Pin1 antibodies with Pin1 proteins shows the lack of green Pin1 staining, demonstrating the specificity of the anti-Pin1 antibody. (G) Immunostaining for Pin1 in 9.5 dpc embryo section reveals Pin1 expression (green) in the embryo. (H) Alkaline phosphatase detection of PGCs (brown, arrowhead) in the adjacent serial-section shows the location of PGCs in the hindgut (hg) at 9.5 dpc. Pin1 protein (green) is expressed in the PGC containing regions of the hindgut in G. (I) Negative control using pre-absorbed anti-Pin1 antibodies shows the lack of green Pin1 staining. (G,I) Red, DAPI stained nuclei. s, somite.

Primordial germ cells express the Pin1 protein

To establish whether germ cells express Pin1, we performed immunohistochemistry using an anti-Pin1 antibody (Winkler et al., 2000). We first observed that Pin1 protein was highly expressed in gonocytes of wild-type mice, with weak expression in Sertoli cells (Fig. 2A). As expected, no Pin1 protein was detected in testis of *Pin1*$^{-/-}$ mice (Fig. 2B). Because postnatal germ cells arise from PGCs, we also investigated whether PGCs express the Pin1 protein. In the gonads of male and female embryos at 13.5 dpc, PGCs, which are identified by their surface antigen PECAM (red, round cells) (Schmahl et al., 2000; Yao et al., 2002), have intense expression of the Pin1 protein as demonstrated by immunofluorescence (green, Fig. 2C,E). The specificity of the Pin1 staining was confirmed using anti-Pin1 antibodies pre-absorbed with Pin1 proteins (Fig. 2D,F). This is consistent with the reported finding that Pin1 is among the genes identified in a 13.5 dpc mixed-sex PGC cDNA library (Abe et al., 1998). To determine whether Pin1 is expressed in PGCs during earlier embryonic development, we performed Pin1 immunostaining in embryo sections, and processed the adjacent serial-sections for alkaline phosphatase assays to identify the regions where PGCs were located. We found that Pin1 is expressed in PGCs as early as 7.5 dpc when they were allocated at the future allantoic bud (see Fig. S2D,E at http://dev.biologists.org/supplemental/). The Pin1 expression is maintained in PGCs during their migration from the base of the allantois at 8.5 dpc to the gonads (see Fig. S2A,B at http://dev.biologists.org/supplemental/). At 9.5 dpc, migrating PGCs, identified by their alkaline phosphatase expression, were seen in the hindgut and dorsal mesentery (Fig. 2H), and were positive for the Pin1 staining (green, Fig. 2G). Negative controls using Pin1 protein pre-absorbed anti-Pin1 antibodies showed no Pin1 staining (Fig. 2I; see Fig. S2C,F at http://dev.biologists.org/supplemental/). These results revealed that Pin1 is expressed in PGCs throughout their embryonic development, suggesting that the absence of *Pin1* might directly affect the development of PGCs in both male and female embryos.

Impaired PGC development in *Pin1*$^{-/-}$ embryos

To investigate the development of primordial germ cells in *Pin1*$^{-/-}$ embryos, we examined embryos and gonads throughout the PGC migratory and proliferative phases, from 8.5 to 13.5 dpc (McLaren, 2000; Tam and Snow, 1981). In *Pin1*$^{-/-}$ embryos at 8.5 dpc, a normal number of PGCs, identified by their high alkaline phosphatase expression (Lawson et al., 1999), was initially allocated at the base of the allantois (Fig. 3A,B). By 9.5 dpc, PGCs were migrating through the hindgut wall in both wild-type and *Pin1*$^{-/-}$ embryos, which were of similar size and developmental stage. However, fewer PGCs were seen in *Pin1*-deficient male and female embryos (Fig. 3C,D). No abnormal or ectopic PGC migration was noted in *Pin1*-deficient embryos, indicating that the PGC number did not decrease as a result of defective migration. On the final day of proliferation, 13.5 dpc, *Pin1*$^{-/-}$ male and female gonads contained fewer PGCs than did wild-type gonads (Fig. 3E-H). Consistent with observations in postnatal testes, the immature testis cords, which are distinguished by the presence of laminin at the basement membrane, formed normally in the *Pin1*-deficient embryonic male gonads (Fig. 3E,F). The overall sizes of *Pin1*$^{-/-}$ embryos and gonads were similar to wild-type, indicating that the difference in the PGC number did not result from general developmental delay.

Because a reduction in the final PGC number could reflect an impairment during the earlier stages of PGC development

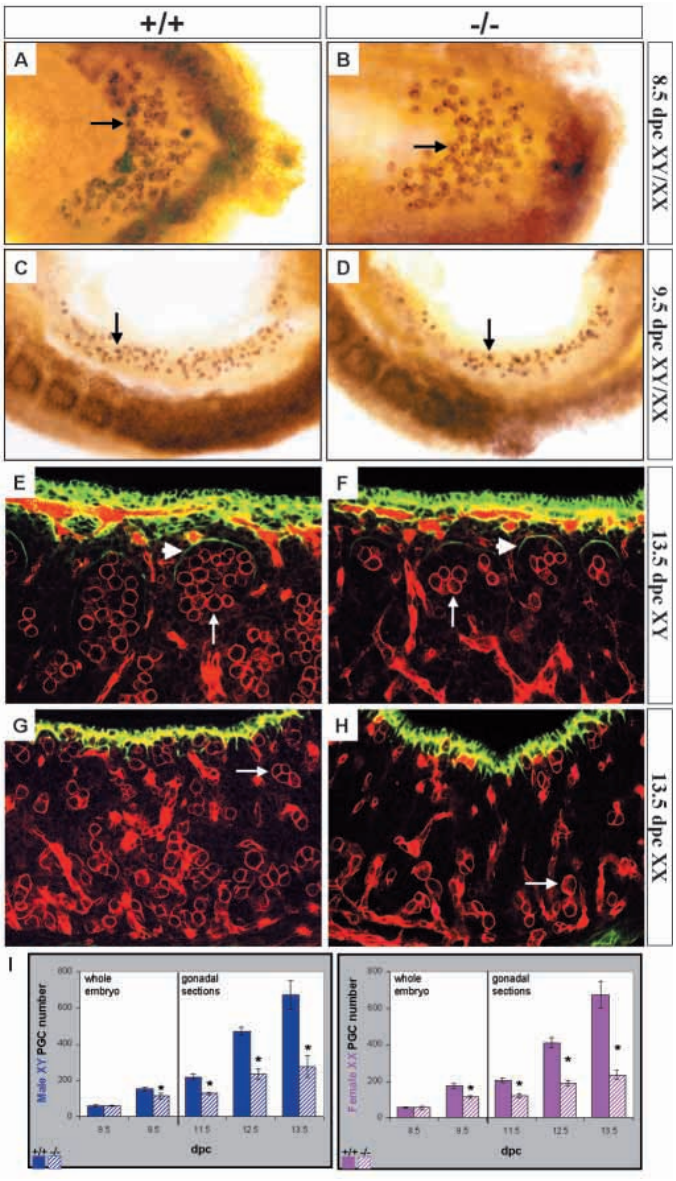

Fig. 3. PGC number is progressively impaired in *Pin1*$^{-/-}$ embryos. (A,C,E,G) Wild-type. (B,D,F,H) *Pin1*$^{-/-}$. (A,B) Light micrograph images of 8.5 dpc wild-type (A) and *Pin1*$^{-/-}$ (B) whole-mount embryos after alkaline phosphatase assays show PGCs (arrow) are properly allocated at the base of the allantois. (C,D) Light micrograph images of 9.5 dpc wild-type (C) and *Pin1*$^{-/-}$ (D) whole-mount embryos after alkaline phosphatase assays show PGCs (arrows) migrating through the hindgut. The *Pin1*$^{-/-}$ embryo has fewer PGCs. (E-H) Confocal images of 13.5 dpc wild-type (E,G) and *Pin1*$^{-/-}$ (F,H) male XY (E,F) and female XX (G,H) gonads after whole-mount immunohistochemistry. PGCs (red, round cells, white arrows) are identified as large round cells by their surface antigen PECAM. Testis cords are identified by laminin at the basement membrane as green crescents (white arrowheads). The *Pin1*-deficient gonads have few PGCs, but normal cords are formed in the male gonad. (I) PGC number in male XY (left, blue) and female XX (right, pink) whole embryos (8.5 and 9.5 dpc) and comparable gonadal sections (11.5-13.5 dpc) at different stages: wild-type, solid; *Pin1*$^{-/-}$, hatched. Values represent mean±s.e.m. *Statistically significant (ANOVA): not significant at 8.5 dpc; $P<0.0005$ at 9.5 dpc; $P<0.0001$ at 11.5-13.5 dpc. Subtests at each age were legitimatised by the genotype × age interaction, $P<0.0001$.

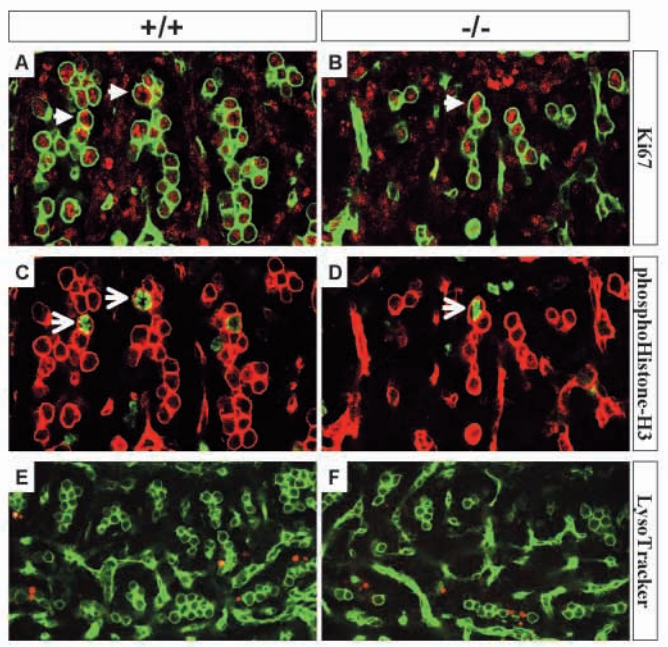

Fig. 4. *Pin1*$^{-/-}$ PGCs do not undergo cell cycle arrest and apoptosis. (A,C,E) Wild type; (B,D,F) *Pin1*$^{-/-}$. (A-F) Confocal images of 12.5 dpc male (XY) gonads after whole-mount immunohistochemistry. (A,B) Nearly all PGCs (green, round cells) are positive for the Ki67 antigen (red, arrowheads) in wild-type (A) and *Pin1*-mutant (B) gonads, indicating that PGCs are actively cycling in the presence and absence of *Pin1*. (C,D) Phosphohistone H3 (green, arrows) identifies PGCs (red, round cells) in mitosis in wild-type (C) and *Pin1*$^{-/-}$ (D) gonads. No accumulation of phosphohistone H3-positive cells is seen in *Pin1*$^{-/-}$ gonads, indicating that *Pin1*$^{-/-}$ PGCs are not arrested in mitosis. (E,F) Wild-type (E) and *Pin1*-mutant (F) PGCs (green, round cells) are not positive for the apoptosis marker LysoTracker (red), indicating that *Pin1*$^{-/-}$ PGCs do not undergo increased apoptosis.

(Lawson et al., 1999), we examined whether PGCs were affected in a continuous or stage-specific manner in *Pin1*$^{-/-}$ embryos. We quantified PGC number in wild-type and *Pin1*-deficient embryos and gonads from 8.5 to 13.5 dpc, and analysed the data using three-factor ANOVA (Fig. 3I). Compared with the increase in PGC number seen in wild-type embryos, PGC number increased significantly more slowly in both *Pin1*-deficient male and female embryos. The progressive nature of the impairment suggested that normal PGC development was disrupted throughout the 5-day period of cell proliferation in *Pin1*$^{-/-}$ embryos, and that *Pin1* is required for expansion of the germ cell lineage after its initial allocation at the base of the allantois.

Absence of cell cycle arrest and apoptosis in *Pin1*$^{-/-}$ PGCs

Because mouse embryo fibroblasts from *Pin1*$^{-/-}$ embryos have been shown to have difficulty entering into the cell cycle from G0 arrest (Fujimori et al., 1999; You et al., 2002), we investigated the cell cycle status of *Pin1*$^{-/-}$ PGCs using antibodies against Ki67, a protein expressed in all phases of the cell cycle but absent in G0 cells (Scholzen and Gerdes, 2000). Almost all PGCs in the wild-type and *Pin1*$^{-/-}$ male and female gonads at 12.5 dpc were positive for the Ki67 antigen

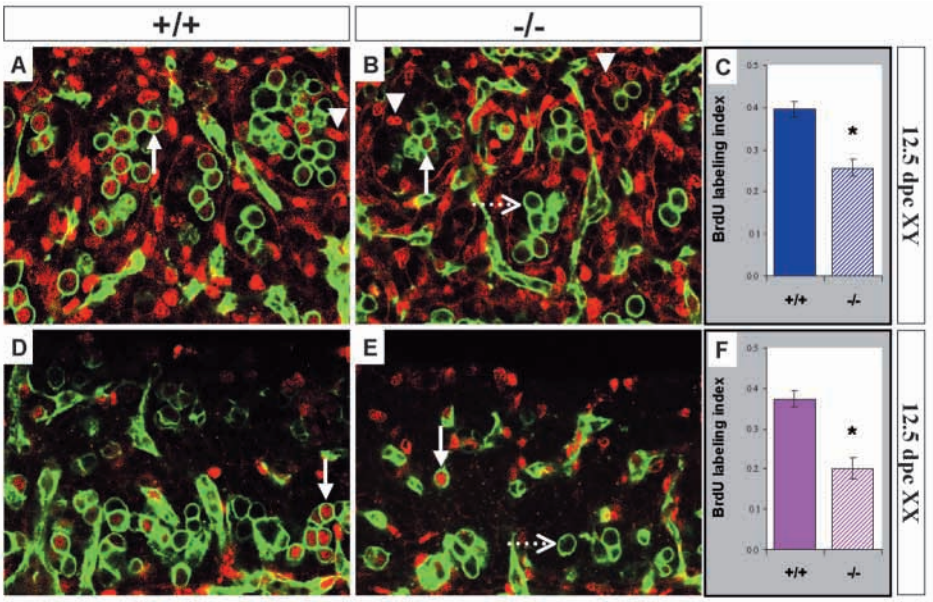

Fig. 5. Decreased proliferation in *Pin1*$^{-/-}$ PGCs. (A-C) Male XY; (D-F) female XX. (A,B,D,E) Confocal images of 12.5 dpc male (A,B) and female (D,E) gonads after whole-mount immunohistochemistry. (A,D) Many PGCs (green, round cells) incorporated BrdU (red, solid arrows) in wild-type gonads. (B,E) Few *Pin1*$^{-/-}$ PGCs were labeled with BrdU (red, solid arrows) in the same period, while many *Pin1*$^{-/-}$ PGCs did not incorporate BrdU (broken arrows). (A,B) *Pin1*$^{-/-}$ Sertoli cells (arrowheads) appeared to be labeled with BrdU similar to wild type. (C,F) Bar graphs of BrdU labeling indices of wild-type (solid) and *Pin1*$^{-/-}$ (hatched) male XY (blue) and female XX (pink) PGCs. Values represent mean±s.e.m. *Statistically significant using unpaired Student's *t*-test, $P<0.001$.

(Fig. 4A,B, male shown), indicating that all PGCs were actively cycling at this stage, and that the absence of *Pin1* did not cause G0 arrest in PGCs. As antisense depletion of Pin1 in HeLa cells, and deletion of the *Pin1* homolog *Ess1* in budding yeast induced mitotic arrest (Lu et al., 1996), we also analysed *Pin1*$^{-/-}$ PGCs using antibodies against phosphohistone H3, a mitosis marker (Fig. 4C,D, male shown). If PGCs in *Pin1*$^{-/-}$ gonads were arrested in mitosis, we would expect to observe a large accumulation of phosphohistone H3-positive cells. However, quantification revealed that the percentage of *Pin1*$^{-/-}$ PGCs in mitosis (6.4%) was not significantly different from wild type (5.8%), indicating that mammalian *Pin1*-deficient PGCs progressed through mitosis. To investigate whether *Pin1* deficiency affected PGC survival, we analysed the presence of apoptotic cells in whole-mount gonads (Yao et al., 2002; Zucker et al., 1999). In 12.5 dpc wild-type gonads, some cells stained positive for the apoptosis marker LysoTracker (red), but little if any overlap was seen with the PGC marker PECAM (green, round cells, Fig. 4E, male shown), consistent with published literature that few PGCs undergo apoptosis at this stage in vivo (Coucouvanis et al., 1993; Yao et al., 2002). Similarly, no apoptotic PGCs were seen in *Pin1*$^{-/-}$ gonads (Fig. 4F, male shown). These findings indicated that unlike cultured cells and budding yeast (Lu et al., 1996), PGCs did not undergo cell cycle arrest and cell death in the absence of *Pin1*.

Decreased proliferation of *Pin1*$^{-/-}$ PGCs

We then assessed the proliferative capacity of PGCs in wild-type and *Pin1*$^{-/-}$ gonads by in vivo 5-bromodeoxyuridine (BrdU) incorporation. Embryos were obtained from heterozygous pregnant females pulse-labeled for 30 minutes with BrdU, which was detected in wholemount gonads (Schmahl et al., 2000). Many PGCs were positive for BrdU in 12.5 dpc wild-type male and female gonads (Fig. 5A,D). By contrast, fewer PGCs incorporated BrdU in the same period in 12.5 dpc *Pin1*$^{-/-}$ male and female gonads (Fig. 5B,E). Quantitative analysis revealed a lower BrdU labeling index in the *Pin1*$^{-/-}$ PGCs in both males and females (Fig. 5C,F). This suggested that the decreased number of PGCs in *Pin1*-deficient embryos was due to decreased cell proliferation. Furthermore, the BrdU labeling of somatic cells (cells other than the round PGCs) was similar in both wild-type and *Pin1*$^{-/-}$ gonads (Fig. 5A,B,D,E). Specifically, Sertoli cells, which can be seen lying along the basement membrane of the testis cords, did not show decreased BrdU incorporation in *Pin1*$^{-/-}$ gonads (Fig. 5A,B), establishing that the proliferative defect in the absence of *Pin1* was germ cell specific.

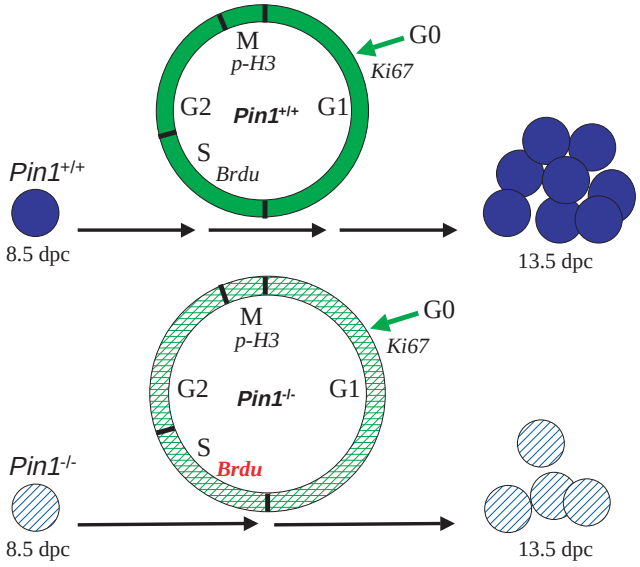

Fig. 6. Illustrative model of cell cycle progression and proliferation in PGCs. Cycling *Pin1*$^{-/-}$ PGCs (bottom) have a lower BrdU labeling index (red), but normal Ki67, phosphohistone H3 and apoptosis marker profiles. This indicates that *Pin1*$^{-/-}$ PGCs have a prolonged cell cycle because of defective cell cycle progression (larger, hatched circle) rather than cell cycle arrest and apoptosis. The net effect of decreased proliferation is fewer cell divisions (represented by arrows) in *Pin1*-deficient PGCs in the same time period, resulting in fewer PGCs at the end of the proliferative phase on 13.5 dpc in the absence of *Pin1*.

DISCUSSION

Based on our results, we propose that *Pin1*-deficient PGCs undergo inefficient cell cycle progression rather than cell cycle arrest, which leads to a lengthening of the cell cycle (Fig. 6, hatched larger circle). The net effect is that *Pin1*$^{-/-}$ PGCs divide less frequently during the 5 day proliferation period compared with wild-type PGCs (Fig. 6, solid smaller circle), resulting in a severely reduced number of germ cells at the end of the proliferative phase (Fig. 6).

From studies in vertebrate cells and yeast it has been proposed that Pin1 plays a role in many aspects of the cell cycle, including G2/M and G1/S progression, G0 re-entry, and mitosis (Crenshaw et al., 1998; Fujimori et al., 1999; Hanes et al., 1989; Lu et al., 1996; Shen et al., 1998; You et al., 2002). The generation of the homozygous *Pin1*-null mice on an isogenic background provided a unique opportunity to investigate the physiological role of Pin1 in the mammal. It has been reported that *Pin1*$^{-/-}$ mice maintained on a mixed genetic background were fertile with normal ovarian morphology and only mild testicular degeneration in older mice (Liou et al., 2002). Here, we show that on an inbred C57BL/6J genetic background, *Pin1* is not only required for normal fertility in both males and females, but also plays a crucial role in primordial germ cell development during mouse embryogenesis.

We have discovered that Pin1 is required for proper cell cycle progression and proliferation of mammalian primordial germ cells in vivo. Interestingly, embryonic somatic cell proliferation did not require *Pin1*, despite the fact that somatic cells also express the Pin1 protein, raising the possibility that embryonic somatic cells possessed an additional compensatory prolyl-isomerase. Recently, it has been reported that another prolyl-isomerase in the parvulin family to which Pin1 belongs, Par14, was upregulated about threefold in *Pin1*$^{-/-}$ MEFs, and inhibitors of both Pin1 and Par14 decreased cell proliferation. Thus, Par14 may function as a compensatory prolyl-isomerase in the absence of Pin1. It is possible that PGCs lack Par14 or that Par14 is not upregulated in PGCs, contributing to the selective germ cell phenotype in *Pin1*$^{-/-}$ mice. Availability of Par14 antibodies would help to investigate this possibility in the future. Alternatively, mammalian PGCs may be particularly sensitive to the loss of *Pin1*. Current evidence suggests that gonadal somatic cells have a sex-specific proliferation pattern that is influenced by the male factor *Sry*, while primordial germ cells appear to have an intrinsic proliferation program that operates in a non-sex-specific manner (Schmahl et al., 2000; Tilmann and Capel, 2002). The fact that *Pin1* regulates both male and female PGC proliferation similarly at 12.5 dpc, after the onset of *Sry*, is consistent with an intracellular function for Pin1 in germ cells, rather than an indirect effect through somatic cells. This view is supported by the high expression of the Pin1 protein in germ cells.

How Pin1 regulates primordial germ cell proliferation and cell cycle progression is currently unknown. Pin1 has a well-recognized function as a mitotic regulator, particularly in cultured transformed cells and in budding yeast (Lu et al., 1996), but its absence did not cause mitotic arrest in mammalian PGCs. However, the mitotic index of *Pin1*$^{-/-}$ PGCs was somewhat higher than wild type, although non-significant with our sampling size. This raises the possibility that the M phase length was prolonged in *Pin1*$^{-/-}$ PGCs, and, if so, *Pin1* may be required for proper progression of PGCs through mitosis. Nevertheless, we favor the idea that PGCs have a prolonged cell cycle as a result of defective G1/S progression in the absence of Pin1. Pin1 has been reported to increase the transcriptional activity of Jun and the stability of cyclinD1, both of which regulate G1/S progression (Liou et al., 2002; Wulf et al., 2001). These studies imply that impaired Jun activity and decreased cyclinD1 levels may contribute to G1/S delays and a lengthening of the cell cycle in *Pin1*-deficient PGCs. However, mice null for phosphorylated Jun (the form that binds Pin1) or cyclinD1 are viable and fertile (Behrens et al., 1999; Fantl et al., 1995), revealing that neither protein can be the sole target responsible for the PGC phenotype and infertility in *Pin1*$^{-/-}$ mice. Therefore, it is possible that Pin1 acts on multiple targets to achieve a combinatorial effect in its regulation of PGC proliferation. Alternatively, an intriguing possibility is that Pin1 may regulate a factor unique to proliferating PGCs. In this regard, in *Drosophila* where Pin1 plays a role in developmental signaling, it regulates the stability of a transcription factor Cf2 in follicle cells in response to growth factor receptor-activated MAPK signaling (Hsu et al., 2001). Of the molecules proposed to influence PGC proliferation, including Scf, Kit and Fgf, many are extracellular growth factors and growth factor receptors capable of activating the MAPK cascade (De Miguel et al., 2002; Godin et al., 1991; Matsui et al., 1992; Resnick et al., 1992; Zhao and Garbers, 2002). Therefore, Pin1 may act as an intracellular signal responder in a growth factor-activated MAPK pathway in primordial germ cells in vivo, ensuring efficient cell cycle progression and facilitating their proliferation to establish the male and female germline.

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